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Ethanolic Extract of *Hedyotis corymbosa* and Its Combination with 5-FU Inhibit Cyclin D Expression on WiDr Colorectal Cancer Cell

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Abstract

Hedyotis corymbosa has been used for long time as an important component in several folklore medicine formula to clinically treat various types of cancer, including colorectal cancer (CRC). Previously, *Hedyotis corymbosa* ethanolic extract (HEE) which contain ursolic acid reported to inhibit CRC growth via induction of cancer cell apoptosis and blocked the cell cycle, preventing G1 to S progression where cyclin D highly espressed in this phase. 5-fluorouracil (5FU), the first line chemotherapy of colorectal cancer have had resistence and possessed several side effects such as neutropenia, immunosuppression, diarrhea, and also constipation. Therefore, the aim of this research is to conduct the antiproliferative effect and molecular analysis of HEE and its combination with 5FU. Molecular docking study was also done to approach the specific protein target of the compound. Antiproliferative effect was conducted by MTT assay, while cyclin D expression was examined by immunofluorescence. The proliferative effect showed that both HEE and 5-FU had cytotoxic effect with IC_{50} value of 65 µg/mL and 90 µM respectively, meanwhile the combination of HEE and 5FU have synergism effect with CI = 0.48 on dose HEE = 22 µg/mL and 5FU = 6.25 µM. Immunofluorescence assay showed HEE and its combination with 5FU suppressed the expression of cyclin D. From molecular docking simulation, ursolic acid performed stable interaction with cyclin D. Our findings suggest that HEE may be an effective treatment for co-chemotherapic for 5-FU through inhibition of cyclin D expression.

Keywords : Hedyotis corymbosa, 5-fluorouracil, colorectal cancer, WiDr, cyclin D

INTRODUCTION

Cancer cells are characterized by uncontrolled proliferation (Hanahan *and* Weinberg, 2011), therefore inhibiting this excessive proliferation is one of the key for the development of anticancer drugs. The transition from G1 phase of the cell cycle to S phase plays crucial role for the control of eukaryotic cell proliferation, and its misregulation promotes oncogenesis. During G1 phase, growth dependent cyclin-dependent kinase (CDK) activity promotes DNA replication and initiates G1 to S phase transition (Bertoli, *et al.*, 2013).

Colorectal cancer is one cancer type which becomes the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women. The prevalence of colorectal cancer has been incresed along the increasing human population (ACS, 2014). First line therapy for CRC, 5-fluorouracil (5FU) has been become resistence despite its side effect like neutropenia, immunosuppression, cardiotoxicity and diarrhea (Denise, *et al.*, 2015; Gressett, *et al.*, 2006; Sorrentino, *et al.*, 2012).

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Recently, natural products have received great interest since they have relatively few side effects compared with modern chemotherapeutics and have been used clinically for thousands of years as significant alternative remedies for a variety of diseases including cancer (Kellof, 2000; Jeune, *et al*., 2005; Won, *et al*., 2006).

One promising medicinal plant is *Hedyotis corymbosa* that belongs to the Rubiaceae family and is widely distributed throughout Souteast Asia. *Hedyotis corymbosa* which contain ursolic acid that has been reported to inhibit the growth of CRC through the induction apoptosis and the blocked the cell cycle on G1/S progression (Suparman, 2008). To further exploration the activity of *Hedyotis corymbosa* ethanolic extract (HEE), we investigated its moleculer mechanism on the proliferation of human colon carcinoma WiDr cells through expression of cyclin D that regulated cell cycle on G1/S progression.

MATERIALS AND METHODS

Sample

The herbs of *Hedyotis corymbosa* were collected from around Universitas Gadjah Mada, Sleman, Yogyakarta. The plant was determined in Biological Pharmacy Labroratorium, Faculty of Pharmacy Universitas Gadjah Mada. The air-dried and ground leaves (300 g) were soaked in ethanol 96% (3 L) for 72 h, then the mixture was filtered to obtain the crude extract. The extract was concentrated using rotary evaporator under vacuum at room temperature to obtain a final residue (23 g) for further experiment. Sample was dissolved in Dimethyl Sulfoxide (DMSO) (Sigma). Both 5 mg/ml 5-FU (Kalbe) and HEE were then diluted in cell culture medium before being applied. DMSO was also used as the co-solvent in dissolving samples in culture medium.

Cell culture

WiDr colorectal cells were obtained from Cancer Cancer Chemoprevention Research Center, Yogyakarta. The cells were grown and maintained in Roswell Park Memorial Institute (RPMI) 1640 (Gibco) supplemented with 10 % $^{v}/_{v}$ Fetal Bovine Serum (FBS) (Sigma) and 1% 10.000 UI/mL Penicillin-Streptomycin at 37^{o}C in humidified atmosphere of 5 % CO_2.

MTT Cytotoxicity Assay

Cell viability was determined by a microculture tetrazolium teachnique (MTT) assay according to the method by Mosmann (1983) with minor modifications. Briefly, WiDr cells were seeded into a 96-well plate (8 x 10^3 cells per well). After 48 h incubation with various concentration of HEE, 5-FU, and its combination, the medium was discarded and added with MTT reagent (0.5 mg/mL) (Biovision) 100µL to each well. The plate was incubated in a humidified atmosphere of 5% CO_2 at 37^{o}C for 4 h and then formazan crystals were dissolved in 100 µL of 0.01 N HCl and incubated in the dark for overnight. The absorbance of cells was measured at 595 nm by a microplate reader. Cells absorbance was converted to % cell viability. Linear regression between concentration and % cell viability giving the equation y = Bx + A were used to calculate IC_{50} value, that is the concentration inhibiting 50% cell proliferation.

Immunofluorescence Assay

Cells (5 x 10^4 cells/well) were seeded on coverslip in 24 well plates until 80% confluent. Cells were treated with fresh medium contain HEE, 5FU, and the combination and incubated in 37^{o}C for 24 h, cells were harvested and were washed using PBS and fixed it with 70% ethanol for 10 min at room temperature. After rinsed again with PBS, cells were incubated with blocking serum 1% BSA for 30 min at room temperature. Then, cells were incubated with primary antibody (anti-cyclin D) for 1 hour at room temperature. After rinsed with PBS, cells were incubated with secondary antibody conjugated by FITC for 1 hour at room temperature in the dark. Then, cell were added by DAPI solution and incubated for 10

min at room temperature in the dark. After rinsed with PBS, cell were added with mounting solution (Fluoromount), put on slide glass, and store at 4°C. The protein expressions were observed under fluorescence microscope.

RESULTS AND DISCUSSIONS

Hedyotis corymbosa, 5-FU, and their combination Inhibited the proliferation of WiDr Cells

Cell viability assay was done to determine the inhibitory effect of HEE and 5-FU single treatment on WiDr cells. The single treatment of 5-FU for 48 h with range of concentration up to 90 μM revealed to possess cytotoxic effect in dose dependent manner. The cytotoxic effect was proven by morphological change on cells (shrinkage and fragmentation). Based on this study, 5-FU had cytotoxic effect by giving IC_{50} value of 90 μM. Interestingly, the treatment of single 5-FU in 24 h did not show significant inhibition on cells proliferation (data not shown), probably because of low sensitivity of WiDr cells toward 5-FU and high expression of timidilat sintetase (TS) on cells (Meyerhardt *and* Mayer, 2005; Giovannetti, *et al.*, 2007). Moreover, 5-FU was also reported to induce NF-κB expression which regulated anti-apoptosis transcription, causing cells were able to escape the apoptosis (Peters, *et al.*, 2002).

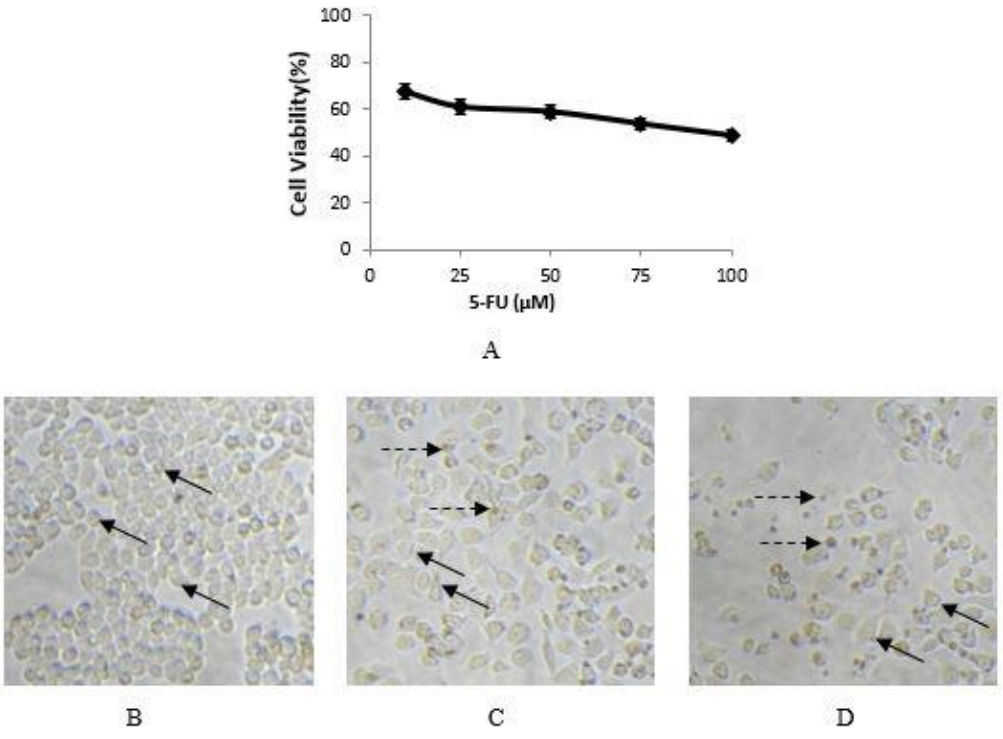

Figure 1. Cytotoxic effect of 5-FU on WiDr cells. Briefly 8x10³ WiDr cells/well were seeded in 96 well-plate and incubated for 24 h before treated with 5-FU for 48 h as described in methods. Cells viability was determined by using MTT assay as described in the methods. (A) Cell viability profile after single treatment of 5-FU for 48 h on WiDr cells. The cells morphological were shown above as (B) cell control, (C) 10 μM of 5-FU, (D) 40 μM of 5-FU. The cells were observe under inverted microscope with 100x magnification. Normal cells were marked as ⟶ meanwhile irregular cells were marked as - - →

The cytotoxic effect of *Hedyotis corymbosa* ethanolic extract (HEE) was also carried out for 48 h, based on several previous studies reported that the bioactive compound in HEE, ursolic acid revealed to give optimum cytotoxic effect after 48 h of treatment (Dong-Kyoo, *et al.*, 2000; Harmand, *et al.*, 2005; Li, *et al*, 2010; Shisodia, *et al.*, 2003). Similar to 5-FU, the treatment with HEE showed inhibitory effect in dose dependent manner; given IC_{50} value of 65 µg/mL. The morphological changes on cells was seen in higher concentration of HEE, indicated that HEE possessed cytotoxic effect toward WiDr cancer cells.

Furthermore, the combination between HEE and 5-FU was carried out on WiDr cells to determine its combination effect on cells proliferation. Based on this study, the treatment of 5-FU and HEE exhibited synergistic effect on WiDr cells, given CI value less than 1, with lowest CI value was showed in combination of 10 µg/mL HEE andd 1.25 µM 5-FU with CI value of 0.33. Based on MTT assay, it was demonstrated that the treatment of HEE in combination with 5-FU inhibited cells proliferation significantly, as the single treatment with 5-FU only inhibit cells proliferation up to 40%, when in combination with 40 µg/mL HEE could decreased cells viability up to 65%. Thus, HEE could be applied as co-chemoterapeutic agent to increase 5-FU sensitivity on colon cancer.

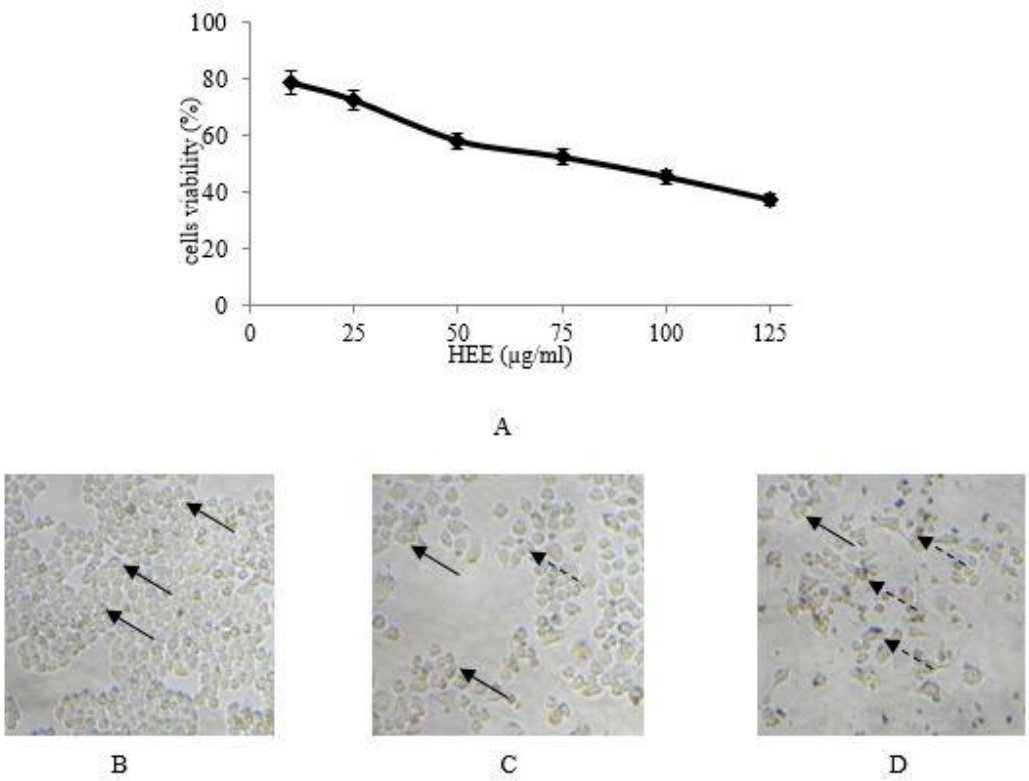

Figure 2. Cytotoxic effect of HEE on WiDr cells. Briefly 8x10³ WiDr cells/well were seeded in 96 well-plate and incubated for 24 h before treated with HEE for 48 h as described in methods. Cells viability was determined by using MTT assay as described in the methods. (A) Cell viability profile after single treatment of HEE for 48 h on WiDr cells. The cells morphological were shown above as (B) cell control, (C) 10 µg/mL of HEE, (D) 40 µM of HEE. The cells were observe under inverted microscope with 100x magnification.

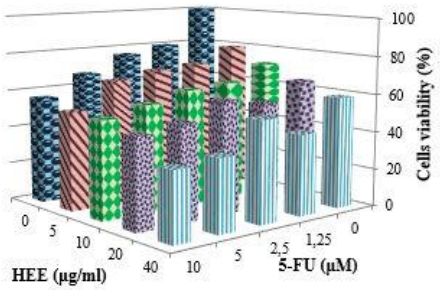

Figure 3. The combination treatment of HEE and 5-FU after 48 h on WiDr cells. Briefly, 10^4 WiDr cells/well were seeded in 96-wellplate before treated with HEE and 5-FU and incubated for next 48 h as described in methods. The effect of combination treatment was determined as % of cells viability (n=3, p<0,05).

Table 1. Combination Index (CI) value from combination treatment of HEE and 5-FU on WiDr cells for 48 h

HEE (µg/ml)	5-FU (µM)			
	1,25	2,5	5	10
5	0.86	0.61	0.82	0.60
10	0.33	0.41	0.48	0.66
20	0.36	0.53	0.50	0.67
40	0.43	0.67	0.51	0.65

Previous study had been reported that HEE caused cell cycle arrest on G1/S phase. Further mechanism has been conducted to evaluate cyclin D expression which regulated cell cycle in G1 to S transition by using immunofluorescence assay.

This research suggests that HEE perform promising potency to be developed as co-chemotherapeutic agent for CRC.

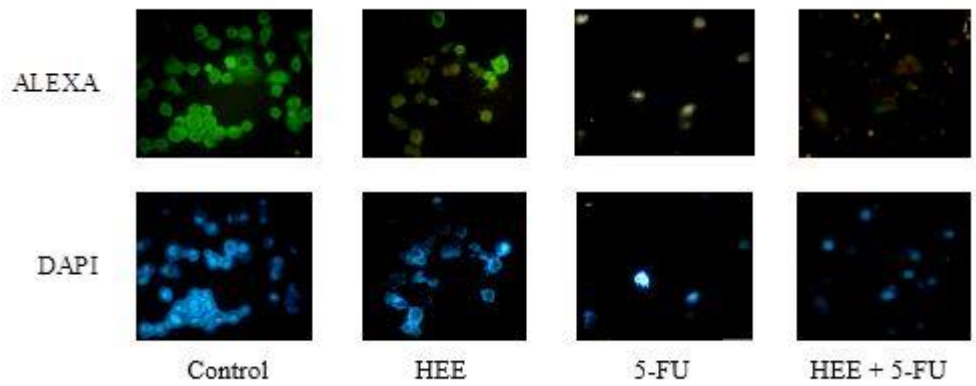

Figure 4. Effect of 5-FU and HEE on cyclin D expression on WiDr Cells. Cells were gorwn on coverslips and treated with compounds as indicated in methods, then subjected to immunofluorescent staining with cyclin D and DAPI as described in materials and methods. Visualizations were done under microscope fluorescent with 400x magnification.

ACKNOWLEDGMENT

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